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Suppression of Lipopolysaccharide-Induced Tumor Necrosis Factor-α Generation from Human Peripheral Blood Monocytes by Inhibitors of Phosphodiesterase 4: Interaction with Stimulants of Adenylyl Cyclase

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SUMMARY

We assessed the role of cyclic nucleotides in modulating lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) generation in human peripheral blood monocytes. Exposure of monocytes to LPS (3 ng/ml) evoked a delayed, time-dependent generation of TNF-α that reached a maximum level 5–6 hr after LPS challenge and remained constant for up to 24 hr. This effect was concentration dependent and resulted in a 20–40-fold increase in the release of TNF-α that was sensitive to actinomycin D and cycloheximide. Treatment of monocytes with agents reputed to activate the cAMP/cAMP-dependent protein kinase (PKA) cascade in general inhibited LPS-induced TNF-α generation. Thus, the β_2-adrenoceptor agonists albuterol and procaterol partially (~40%) suppressed TNF-α generation in a propranolol-sensitive manner. Furthermore, 8-bromo-cAMP, cholera toxin, prostaglandin E_2, and a number of drugs (i.e., rolipram (ZK 62711), denbufylline (BRL 30892), Ro 20–1724, benafentrine (AH 21–132), that inhibit the phosphodiesterase (PDE) 4 isoenzyme family abolished cytokine generation. In contrast, forskolin, inhibitors of PDE3 and PDE5, and activators of soluble and particulate guanylyl cyclase were essentially inactive. Interestingly, rolipram failed to potentiate the inhibitory effect of albuterol on LPS-induced TNF-α biosynthesis but, paradoxically, synergized with albuterol in the generation of cAMP and in the activation of PKA. When PGE_2 was used to activate adenylyl cyclase, however, rolipram potentiated cAMP accumulation, PKA activation, and inhibition of TNF-α generation. In contrast, forskolin did not increase the cAMP content of monocytes in the absence or presence of rolipram. Collectively, these data suggest that LPS-induced TNF-α generation by human peripheral blood monocytes is due to increased transcription and subsequent translation of the TNF-α gene and that these effects are suppressed by a range of agents that activate the cAMP/PKA cascade. However, the failure of rolipram to potentiate the inhibitory effect of albuterol and procaterol on TNF-α generation suggests that β_2-adrenoceptor agonists may affect gene expression and/or post-transcriptional regulatory processes by, at least in part, a cAMP-independent mechanism(s).

TNF-α is a pleiotropic 17-kDa polypeptide cytokine that is one of the most abundant products secreted by activated macrophages and monocytes (1). In general, TNF-α may be considered proinflammatory, and it has been implicated in the pathogenesis of a number of diseases, including the acute syndromes associated with endotoxin shock and bacterial sepsis, glomerular nephritis, pancreatitis, rheumatoid arthritis, and multiple sclerosis (2). More recently, persuasive evidence has emerged that TNF-α may play an important role in initiating and/or perpetuating some of the chronic manifestations of allergic asthma (3–8). TNF-α and TNF-α mRNA levels are elevated in a number of other cells resident in lung tissue (e.g., macrophages, monocytes, mast cells, T lymphocytes, epithelial cells) after activation of the IgE receptor *in vitro* (3–5), and there is evidence of enhanced ex-

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ABBREVIATIONS: PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PGE_2, prostaglandin E_2; SNP, sodium nitroprusside; DTT, dithiothreitol; ANP, atrial natriuretic peptide, TEA, triethanolamine; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; HBSS, Hanks' balanced salt solution; IP_{20}, protein kinase inhibitor peptide (TTYADFIASGRTGRRNAIHD); Percoll, polyvinylpyrrolidone-coated silica gel; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; BSA, bovine serum albumin; IL, interleukin; mRNA, messenger RNA; denbufylline, 1,3-di-*n*-butyl-7-(2-oxo-propyl)xanthine); SK&F 95654, (5-methyl-6-[4,4-oxo-1,4-dihydropyridin-1-yl)phenyl]-4,5-dihydro-3(2*H*)-pyridazinone; SK&F 96231, 2-(2-propoxyphenyl)-6-purinone; rolipram, 4-(3-cyclo pentyloxy-4-methoxy phenyl)-2-pyrrolidine); zaprinast, 2-*O*-propoxyphenyl-8-azapurin-6-one; benafentrine (AH 21–132), (±)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methyl-benzo-[c(1,6)]naphthyridine; MY 5445, 1-(3-chlorophenylamino)-4-phenyl-phthalazine; Ro 20–1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolinone.

pression of TNF-α mRNA in asthmatic airways (6). *In vivo*, it has been documented that the amount of TNF-α in bronchoalveolar lavage fluid recovered from patients with symptomatic asthma is significantly increased relative to that obtained from asthmatic individuals who are apparently asymptomatic (3). Moreover, exposure of asthmatic subjects to allergen evokes the release of TNF-α from alveolar macrophages at a time that coincides with the development of the late phase response (7). Also, a recent study (8) has found that inhalation of TNF-α increases human airway responsiveness to methacholine and promotes pulmonary neutrophil recruitment. Taken together, these are intriguing observations that may relate to the ability of TNF-α to promote the generation of other proinflammatory chemotactic cytokines [e.g., IL-8, granulocyte/macrophage colony-stimulating factor (9)] and to up-regulate the adhesion molecules intercellular adhesion molecule-1 (10) and vascular adhesion molecule-1 (11) on airway epithelial cells and on the microvascular endothelium, respectively.

Given the potential role of TNF-α in inflammation, a number of approaches are being evaluated toward the control of the elaboration and pathological actions of this cytokine. One of these, which has proved to be experimentally and clinically efficacious, is the alkylxanthine pentoxifylline, which prevents TNF-α generation in laboratory animals and in human volunteers with experimental endotoxemia (12–14). The mechanism underlying the beneficial effects of pentoxifylline is not unequivocally established but may be attributable to its ability to act as a cAMP PDE inhibitor (15). This is an important observation because there has been an extraordinary resurgence of interest in the potential usefulness of PDE inhibitors for the treatment of a number of chronic inflammatory diseases, in particular, asthma (16–19). This has arisen for several reasons. It is now recognized that cyclic nucleotide PDE is not a single enzyme but rather a generic term used to describe a diverse group of structurally distinct proteins that, based on biochemical characteristics and primary sequence analyses of partial and full-length complementary DNA clones, have been broadly categorized into seven isoenzyme families (PDE1, Ca^{2+}/calmodulin dependent; PDE2, cGMP stimulated; PDE3, cGMP inhibited; PDE4, cAMP specific; PDE5, cGMP specific; PDE6, photoreceptor; PDE7, rolipram insensitive, cAMP specific) (20). *In vitro* studies have established that selective inhibitors of the PDE4 and, to some extent, the PDE3 isoenzyme families suppress many functional indices of cell activation that are considered proinflammatory (16–19). Furthermore, the complement of PDE isoenzymes expressed by different cell types varies, a finding that could theoretically be exploited with selective inhibitors targeted against the desired PDE isoenzyme that is present in the cell of interest. This possibility is being actively pursued by the pharmaceutical industry for the treatment of asthma on the premise that essentially all proinflammatory and immunocompetent cells express exclusively or predominantly one or more members of the PDE4 isoenzyme family that, in many cases, are the sole regulators of cAMP degradation (16–19). Collectively, therefore, it is hoped that PDE4 inhibitors will exhibit an improved side-effect profile over nonselective drugs (e.g., theophylline, enprofylline) that indiscriminately inhibit all PDE isoenzymes.

In view of the therapeutic potential of PDE inhibitors in allergic inflammation and the apparent significance of TNF-α in asthma pathogenesis, we conducted experiments to (a) assess the ability of representative inhibitors of the PDE3, PDE4, and PDE5 isoenzyme families to suppress TNF-α generation by human peripheral blood monocytes; (b) compare the inhibitory effect of PDE inhibitors on TNF-α production with that of other drugs reputed to increase cyclic nucleotide levels in intact cells; and (c) determine the roles of cAMP and PKA in modulating TNF-α production.

Materials and Methods

Isolation and purification of human mononuclear cells. Blood was collected from normal healthy individuals by antecubital venipuncture into acid citrate dextrose (160 mM disodium citrate, 110 mM glucose, pH 7.4) and mixed with 6% w/v hydroxymethyl starch to sediment erythrocytes. After standing the mixture at room temperature for 90 min, the leukocyte-rich plasma was removed and centrifuged at 312 × *g* for 7 min. The resulting cell pellet was gently resuspended in approximately 7 ml of buffer A (5 mM KH_2PO_4, 5 mM K_2HPO_4, 110 mM NaCl, pH 7.4) with 50% v/v with Percoll and layered over a discontinuous Percoll density gradient [63:73% (v/v)] in buffer A. Mononuclear cells were subsequently separated from polymorphonuclear cells by centrifugation at 1200 × *g* for 30 min at 18°. With this procedure, mononuclear cells were recovered from the 55:70% (v/v) Percoll interface.

Mononuclear cells were washed twice in Ca^{2+}/Mg^{2+}-free HBSS to remove Percoll and finally suspended in Ca^{2+}/Mg^{2+}-free HBSS at a concentration of 10^6 cells/ml. Cells (5×10^5) were added to 24-well culture plates (Greiner Labortecnik, Dursley, Gloucestershire, UK) containing 500 μl Dutch-modified RPMI 1640 (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) and allowed to adhere to the plastic for 90 min at 37° in a humidified incubator under an atmosphere of 5% CO_2. Plates were agitated, nonadherent cells were decanted, and the resulting monocytes were cultured in 1 ml Dutch-modified RMPI 1640 in the absence and presence of LPS (100 pg/ml to 1 μg/ml) with or without varying concentrations of the compounds indicated in the text and figure legends. The ability of a range of pharmacological agents to modify the release of TNF-α into the culture supernatant evoked by LPS was subsequently measured with an ELISA (see below). The purity of the adherent cell population was assessed with Kimura staining and was routinely >97%.

Measurement of TNF-α. TNF-α released from cultured monocytes was measured with an amplified sandwich ELISA. Round-bottom plates (96-well plates; Greiner Labortecnik Ltd) were coated with 100 μl of a mouse anti-human TNF-α monoclonal antibody diluted 1:400 in buffer B (15 mM Na_2CO_3, 36 mM $NaHCO_3$, 15 mM NaN_3, pH 9.6) and left for 2 hr at 37°. Plates were subsequently washed in buffer C (145 mM NaCl, 4 mM KCl, 10 mM NaH_2PO_4, 3 mM Na_2HPO_4, 0.05% v/v Tween-20, pH 7.4) and immediately treated with BSA (5% w/v) for 30 min at 37°. After an additional wash with buffer C, 100 μl TNF-α standards, quality controls, and unknown samples (diluted 1:4 in buffer C) were added to the plates and left for 18 hr at 4°. Plates were washed in buffer C, incubated for 2 hr with 100 μl of a rabbit anti-human polyclonal TNF-α antibody (diluted 1:500 in buffer C supplemented with 10% v/v FCS), washed again, and then incubated for an additional 2 hr at room temperature with 100 μl of an alkaline phosphatase-labeled sheep anti-rabbit polyclonal IgG antibody (diluted 1:2000 in buffer C supplemented with 10% FCS). Plates were washed again and developed with a *p*-nitrophenyl-phosphate assay kit (code 50–80-00, KPL/Dynatech Laboratories, Billingshurst, Sussex, UK) according to the manufacturer's instructions. TNF-α was measured colorimetrically at 405 nm and quantified by interpolation from a standard curve constructed to known concentrations of human recombinant TNF-α. The detection limit of this assay is 8 pg/ml.

Measurement of cyclic nucleotide PDE activity. PDE activity was measured using a modification of the method described by Dent *et al.* (21). Adherent monocytes were lysed in ice-cold buffer D (20 mM TEA, pH 8, 1 mM EDTA, 1% v/v Triton X-100) supplememted with the proteinase inhibitors benzamidine (2 mM), leupeptin (50 μM), PMSF (100 μM), bacitracin (100 μg/ml), and soybean trypsin inhibitor (10 μg/ml). Aliquots (30 μl) of the resulting lysate were added to 270 μl of a reaction medium containing (final concentration) 20 mM TEA, pH 8.0, 5 mM DTT, 500 μg/ml BSA, 5 mM magnesium acetate, 0.25 unit alkaline phosphatase, 1 mM EGTA, 1 μM cAMP (supplemented with ~250,000 dpm of [8-^{3}H]cAMP and ~5,000 dpm [8-^{14}C]adenosine to estimate recovery) and the drug under evaluation or its vehicle. The reaction was terminated by the addition of 1 ml of a mixture of Dowex AG 1X8/methanol/water (1:2:1), vortex mixed, and placed into an ice bath until the end of the assay. Samples were then vortex mixed for an additional 30 min before being centrifuged at 12,000 × *g* for 5 min at 4°. The radioactivity in 750-μl aliquots of the resulting supernatants was determined by liquid scintillation counting in 2 ml ACS II scintillant (Amersham) at a counting efficiency of ~60%. PDE activity is expressed as the formation of nucleoside 5′ monophosphate from cAMP or cGMP/min/10^{6} cell equivalents (i.e., the lysate derived from 10^{6} cells) at 37° after correction for the recovery (~65–85%) of [8-^{14}C]-adenosine.

Classification of cyclic nucleotide PDEs. Cyclic nucleotide PDEs are classified according to the new nomenclature outlined in Beavo *et al.* (22). Thus, *HS*PDE1, *HS*PDE2, *HS*PDE3, *HS*PDE4, *HS*PDE5, *HS*PDE6, and *HS*PDE7 refer to the Ca^{2+}/calmodulin-dependent, cGMP-stimulated, cGMP-inhibited, cAMP-specific, cGMP-specific, photoreceptor, and rolipram-insensitive/cAMP-specific PDE families, respectively, where the prefix, *HS*, refers to the species *Homo sapiens*.

Measurement of cAMP. Adherent monocytes (10^{6} cells/well) were treated with the drugs under investigation, and at the appropriate time points, as indicated in the text and figure legends, the supernatants were decanted and the cells were lysed by the addition of 500 μl hot (~90%) trichloroacetic acid (1 M). The contents of each well were aspirated into polypropylene Eppendorf tubes and centrifuged at 12,000 × *g* for 5 min to precipitate cell debris, and the resulting supernatants were subsequently neutralized. Aliquots (500 μl) of the neutralized extracts were acetylated by the consecutive addition of triethylamine (20 μl) and acetic anhydride (10 μl), and cAMP mass was measured immediately by radioimmunoassay. Briefly, to 200 μl of acetylated sample was added 50 μl of adenosine-3′,5′-monophospho-2-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (~2000–3000 dpm) in 0.2% BSA and 100 μl of anti-cAMP antibody in 0.2% BSA. After vortex mixing, samples were incubated overnight at 4°, and free and antibody-bound cAMP was separated by charcoal precipitation with ice-cold potassium phosphate buffer (100 mM in 0.2% BSA, pH 7.4) and quantified by γ-counting. The detection limit and sensitivity (IC_{50}) of this assay are 10 and 145 fmol, respectively.

Measurement of the PKA activity ratio. Adherent monocytes (5 × 10^{5} cells/well) were treated with the drugs under investigation, and at the appropriate time points, as indicated in the text and figure legends, the supernatants were decanted and the cells were lyzed by the addition of 500 μl ice-cold buffer E [20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 125 mM KCl, 5 mM DTT, 2% (v/v) Triton X-100]. The contents of each well were aspirated into polypropylene Eppendorf tubes and centrifuged at 12,000 *g* for 5 min to precipitate cell debris, and the resulting supernatants used immediately as the enzyme source.

PKA activity was measured with a modification of the method of Giembycz and Diamond (23). Assays were performed in duplicate at 30° in borosilicate glass tubes and initiated by the addition of 25 μl of monocyte extract to 75 μl of a reaction mixture containing (final concentration) 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 10 mM magnesium acetate, 5 mM DTT, 100 μM 3-isobutyl-1-methylxanthine, 2 mg/ml BSA, 100 μM ATP (supplemented with ~100 cpm/pmol [γ-^{32}P]ATP), and 100 μM Kemptide in the absence and presence of 10 μM cAMP. In all cases, phosphotransferase activity was defined with IP_{20} (1 μM), a synthetic icosapeptide inhibitor of PKA (23). Reactions were terminated after 60 min by pipetting 70-μl aliquots of the mixture onto 2 × 2-cm phosphocellulose paper squares (Whatman P 81), which were left for 30 sec before being immersed in 150 mM phosphoric acid. The paper squares were extensively washed in fresh phosphoric acid, immersed in ethanol and diethylether, and allowed to dry. Bound radioactivity (representing phosphoKemptide) was subsequently quantified by liquid scintillation counting. Under these conditions, <5% of the substrate was used. One unit of phosphotransferase activity was defined as the amount of PKA that catalyzed the incorporation of 1 nmol γ-phosphate from ATP into Kemptide/min/10^{6} cell equivalents at 30°. Unless stated otherwise, PKA is expressed as an activity ratio that is defined as the specific activity of PKA measured in the absence of exogenous cAMP divided by the specific activity of PKA obtained in the presence of sufficient exogenous cAMP to maximally activate the enzyme.

Drugs and analytical reagents. The following were purchased from Sigma Chemical Co. (Poole, Dorset, UK): cAMP, cGMP, calmodulin (code P 2277), 8-bromo-cAMP, 8-bromo-cGMP, alkaline phosphatase (code P 4252), bacitracin, benzamidine, soybean trypsin inhibitor, leupeptin, PMSF, Dowex AG 1X8 (200–400 mesh, chloride form), Wright-Giemsa, cholera toxin, TEA, Kemptide, IP_{20}, albuterol, procaterol, propranolol, Kimura stain, dexamethasone, cycloheximide, actinomycin D, PGE_2, forskolin, SNP, ANP, LPS (from *Salmonella enteritis*), and flurbiprofen. FCS, RPMI 1640, and HBSS were obtained from Flow Laboratories (Rickmansworth, Hertfordshire, UK), Percoll was obtained from Pharmacia/LKB (Milton Keynes, Buckinghamshire, UK), and charybdotoxin (from *Leiurus quinquestriatus*) was purchased from Peninsula Laboratories (St. Helens, Merseyside, UK). Denbufylline, SK&F 95654, and SK&F 96231 were donated by SmithKline-Beecham (Harlow, Middlesex, UK). *RS*-rolipram, zaprinast (M&B 22,948), and *RS*-benafentrine were kindly provided by Schering AG (Berlin, Germany), Rhone-Poulenc Rorer Ltd (Dagenham, Essex, UK), and Sandoz AG (Basel, Switzerland), respectively. MY 5445 was purchased from Calbiochem (Nottingham, UK), and Ro 20–1724 was purchased from Semat (St. Albans, Hertfordshire, UK). [8-^{3}H]cAMP (0.74–1.1 TBq/mmol), [8-^{3}H]cGMP (0.37–1.1 TBq/mmol), [γ-^{32}P]ATP (0.74–1.5 TBq/mmol), [8-^{14}C]adenosine (1.85–2.2 GBq/mmol), and adenosine-3′,5′-monophospho-2′-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (~74 TBq/mmol) were obtained from Amersham International (Buckinghamshire, UK). TNF-α was obtained from British Biotechnology (code BDP 28) and the National Institute for Biological Standards and Controls (code 87/650) for standards and quality controls, respectively. Mouse anti-human TNF-α, rabbit anti-human TNF-α, and alkaline phosphatase-labeled sheep anti-rabbit IgG antibodies were purchased from Serotec (Kidlington, Oxford, UK), Genzyme Corporation (West Malling, Kent, UK), and Stratech Scientific (Luton, Bedfordshire, UK), respectively. All other reagents were from BDH (Poole, Dorset, UK).

Dissolution and storage of drugs. Stock solutions were made at a concentration of 100 mM in aqueous media (cholera toxin, albuterol, procaterol, flurbiprofen, benafentrine, ANP, SNP, 8-bromo-cAMP, 8-bromo-cGMP, Kemptide, IP_{20}), absolute ethanol (denbufylline, rolipram, Ro 20–1724, DMSO (MY 5445, SK&F 95654, SK&F 96231, forskolin), or 0.5 M NaOH (zaprinast). Charybdotoxin (10 μM stock solution) was dissolved in 0.9% (w/v) NaCl containing 0.1% (w/v) BSA. Drugs were diluted to the desired working concentration in enriched Dutch-modified RPMI 1640. Human recombinant TNF-α for both quality controls and standards was obtained as a lyophilize powder and reconstituted at 1 mg/ml in distilled water and stored at −70°. When required, TNF-α was diluted in enriched Dutch-modified RPMI 1640.

Data and statistical analyses. Data points and values in the text and figure legends represent the mean ± standard error of *n* independent determinations. Concentration-response curves were

analyzed by least-squares nonlinear iterative regression with the PRISM curve-fitting program (GraphPad software, San Diego, CA). IC_{50} values refer to the concentration of drug required to inhibit TNF-α release and cAMP hydrolysis by 50%, whereas EC_{50} values represent the concentration of drug required to produce a response that is 50% of the maximum attained by that drug. Where appropriate, data were analyzed parametrically using the statistical package Number Cruncher Statistical System (Kaysville, Utah). Student's *t* test (two-tailed) or one-way ANOVA/Newman-Keuls test was used to assess significance between control and treatment groups. The null hypothesis was rejected when $p < 0.05$.

Results

Complement of cyclic nucleotide PDEs in human monocytes. Human peripheral blood monocyte lysates hydrolyzed cyclic purine nucleotides with a preference for cAMP over cGMP (1.86 ± 0.12 versus 0.08 ± 0.05 pmol/min/10^6 cell eq. at 1 μM substrate; Fig. 1a). cAMP PDE activity was slightly increased (~16%) by calmodulin (25 units plus 2 mM $CaCl_2$) but was unaffected by cGMP (10 μM), suggesting that these cells express a small amount of an *HS*PDE1 but no detectable *HS*PDE2 or *HS*PDE3 (Fig. 1a). cAMP hydrolysis was also inhibited in a concentration-dependent manner by rolipram, denbufylline, and Ro 20–1724 with respective IC_{50} values of 0.89 ± 0.06, 1.37 ± 0.23, and 6.89 ± 0.84 μM. In each case, 80–95% inhibition of hydrolysis was achieved at the highest concentration of drug examined, indicating that the predominant cAMP PDE in human monocytes was an *HS*PDE4. Benafentrine, a mixed PDE3/PDE4 inhibitor, similarly suppressed cAMP PDE activity (IC_{50} = 5.23 ± 0.94 μM), whereas the PDE3 inhibitor SK&F 95654 was inactive except at concentrations of >30 μM, where selectivity for *HS*PDE3 is lost (Fig. 1b).

The concentration-response curves that described the inhibition of cAMP hydrolysis by rolipram, denbufylline, and Ro 20–1724 were shallow, with pseudo-Hill coefficients (−0.504 ± 0.015, −0.548 ± 0.037, and −0.626 ± 0.053, respectively) significantly less than unity (Fig. 1b). Benafentrine, in contrast, inhibited *HS*PDE4 with a slope of −0.969 ± 0.055 (Fig. 1b). Differential centrifugation (45,000 × *g*, 30 min) of monocyte lysates revealed that 94.3 ± 5.9% of the cAMP PDE activity was recovered in the soluble fraction (data not shown; five experiments).

Effect of LPS on TNF-α biosynthesis. The basal amount of TNF-α released by human peripheral blood monocytes after they had been cultured for 18 hr in enriched RPMI 1640 was low (20–200 pg/ml). However, treatment of these cells with LPS (100 pg/ml to 1 μg/ml) resulted in a concentration-dependent stimulation of TNF-α generation with an EC_{50} of 238 ± 12.1 pg/ml (Fig. 2). The maximally effective concentration of LPS was 10–100 ng/ml and was associated with a 20–40-fold increase in the amount of immunoreactive TNF-α (~1.5–3 ng/ml) recovered from the culture supernatant at 18 hr (Fig. 2). Unless stated otherwise, LPS was used in all further experiments at a submaximal concentration of 3 ng/ml that equated to an EC_{84} for TNF-α biosynthesis (Fig. 2).

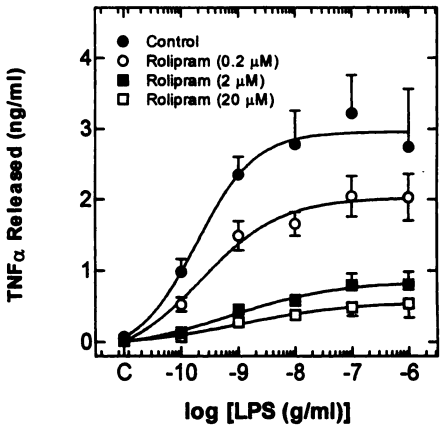

Fig. 2. Concentration-response curves of LPS-induced TNF-α biosynthesis from human peripheral blood monocytes in the absence and presence of the PDE4 inhibitor rolipram. Adherent monocytes were pretreated for 20 min with rolipram or its ethanol vehicle (●, 0.0001% v/v) before being exposed to varying concentrations of LPS (100 pg/ml to 1 μg/ml). Cells were maintained at 37° in a thermostatically controlled incubator under a 5% CO_2 atmosphere, and the amount of TNF-α released into the culture supernatant was measured at 18 hr with a sandwich ELISA as detailed in Materials and Methods. Values represent the mean ± standard error of three determinations made from different cell preparations.

Fig. 3 shows the time course of TNF-α generation. After the addition of LPS, there was a lag period of ~1 hr before an increase in TNF-α-like immunoreactivity was detected in the culture supernatant. Thereafter, the release of TNF-α increased with time ($t_{1/2}$, ~2.8 hr) and reached a maximum 5–6 hr after LPS challenge that remained constant for up to 24 hr (Fig. 3). In all subsequent experiments, TNF-α was measured 18 hr after the addition of LPS.

Effect of flurbiprofen, cycloheximide, actinomycin D, and dexamethasone on LPS-induced TNF-α generation. LPS has been reported to promote the generation of PGE_2 in phagocytic cells from a number of species that may act in an autocrine manner to suppress TNF-α biosynthesis (24). In this study, however, pretreatment of monocytes with the cyclo-oxygenase inhibitor flurbiprofen (1 μM) did not affect the generation of TNF-α evoked by any concentration (0.1, 0.3, or 3 ng/ml) of LPS examined (data not shown).

Exposure of human monocytes for 5 and 20 min to either actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml), inhib-

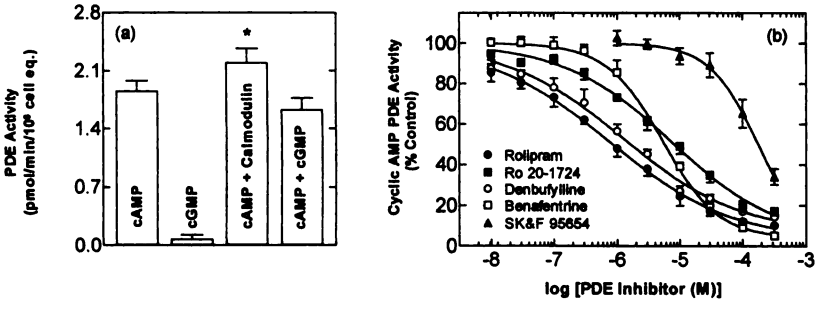

Fig. 1. Effect of allosteric modulators and isoenzyme-selective PDE inhibitors on cyclic nucleotide hydrolysis in lysates of human peripheral blood monocytes. Adherent monocytes were subjected to osmotic lysis, and cyclic nucleotide hydrolysis in aliquots of the resulting lysate was measured in the absence and presence of (a) cGMP (10 μM), Ca^{2+} (2 mM)/calmodulin (25 units) and (b) a number of selective PDE inhibitors. Experiments were conducted at a substrate concentration of 1 μM. Values represent the mean ± standard error of three determinations made from different cell preparations. *, $p <$ 0.05, significant enhancement of cAMP hydrolysis.

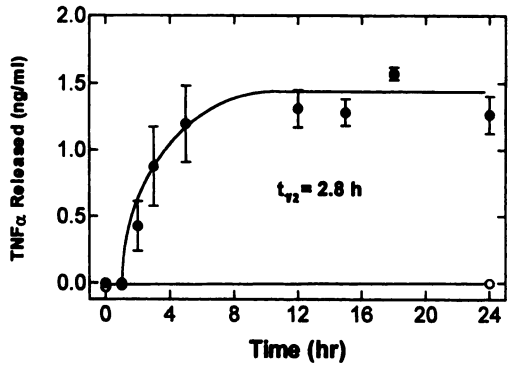

Fig. 3. Time course of LPS-induced TNF-α biosynthesis from human peripheral blood monocytes. Adherent monocytes were cultured in the absence (○) and in the presence (●) of a submaximal concentration of LPS (3 ng/ml; EC_{84}), and the amount of TNF-α released in to the culture supernatant was measured at the time points indicated by a sandwich ELISA. Values represent the mean ± standard error of four determinations made from different cell preparations. See Fig. 2 legend and Materials and Methods for further details.

itors of transcription and translation, respectively, before the addition of LPS abolished TNF-α biosynthesis (data not shown). Similarly, pretreatment of cells for 20 min with the glucocorticosteroid dexamethasone suppressed LPS-induced TNF-α biosynthesis in a concentration-dependent manner with an IC_{50} of 2.9 ± 1.1 nM (Fig. 4). Interestingly, ~20% of the LPS-induced response was insensitive to dexamethasone (Fig. 4).

Effect of rolipram on the time course of LPS-induced TNF-α generation. Pretreatment of monocytes for 20 min with 0.2, 2, or 20 μM concentrations of the PDE4 inhibitor rolipram produced a "mixed" inhibition of LPS-induced TNF-α generation (Fig. 2). Thus, there was a progressive, concentration-related reduction in the maximal response elicited by LPS that was associated with a modest, but commensurate, reduction in the potency of LPS (EC_{50} values: 0.56 ± 0.06, 1.48 ± 0.13, and ~2 ng/ml for 0.2, 2, and 20 μM rolipram, respectively).

Effect of isoenzyme-selective PDE inhibitors on LPS-induced TNF-α generation. The suppressive effect of rolipram on LPS-induced TNF-α generation described was evaluated in more detail. As shown in Fig. 5a, rolipram inhibited cytokine production in a concentration-dependent manner. Curiously, the rolipram concentration-response curve was biphasic, with IC_{50} values of ~75 nM and 35 μM for the high- (79%) and low- (21%) potency components, respectively (Fig. 5a; Table 1). Denbufylline (PDE4/PDE5 inhibitor), Ro 20–1724 (PDE4 inhibitor), and benafentrine (PDE3/PDE4 inhibitor) similarly abolished TNF-α generation with the same rank order of potency as for their inhibition of cAMP hydrolysis. In contrast to rolipram, however, their concentration-response curves were monophasic (Fig. 5b). It is noteworthy that the slope of the concentration-response curves for the inhibition of TNF-α generation by denbufylline and Ro 20–1724 (but not benafentrine) were shallow, with pseudo-Hill coefficients significantly less than unity (Table 1).

The PDE3 inhibitor SK&F 95654 and the PDE1/PDE5/PDE6 inhibitors zaprinast, MY 5445, and SK&F 96231 did not significantly affect LPS-induced TNF-α generation at concentrations where isoenzyme selectivity is preserved (Fig. 5b; Table 1).

Effect of cell-permeant cyclic nucleotide analogues and of cholera toxin on LPS-induced TNF-α generation. Exposure of monocytes for 20 min to 8-bromo-cAMP inhibited LPS-induced TNF-α generation in a concentration-dependent manner with an IC_{50} value of 123.5 ± 14.4 μM (Fig. 6a). Maximum inhibition was achieved at 3 mM 8-bromo-cAMP, a concentration that abolished cytokine generation. 8-Bromo-cGMP elicited qualitatively identical results but was ~8-fold less potent (IC_{50} = 1.05 ± 0.54 mM; Fig. 6a).

Pretreatment of human adherent monocytes for 2 hr with cholera toxin before the addition of LPS also attenuated the amount of immunoreactive TNF-α released into the culture supernatant at 18 hr. This effect was concentration related (IC_{50} = 155.2 ± 61.3 pg/ml), with >98% inhibition achieved at 10 ng/ml cholera toxin (Fig. 6b).

Effect of β_2-adrenoceptor agonists PGE_2 and forskolin on LPS-induced TNF-α generation: Interaction with rolipram. The finding that inhibitors of the PDE4 isoenzyme family, cholera toxin and 8-bromo-cAMP, inhibited LPS-induced TNF-α biosynthesis prompted further studies to assess whether agonists that increase cAMP via an interaction with their cell surface receptors also exert a sup-

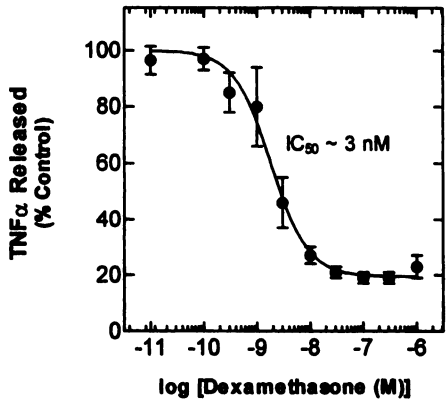

Fig. 4. Inhibitory effect of dexamethasone on LPS-induced TNF-α biosynthesis from human peripheral blood monocytes. Adherent monocytes were pretreated for 20 min with varying concentrations of the glucocorticosteroid dexamethasone before being exposed to LPS (3 ng/ml; EC_{84}). Values represent the mean ± standard error of six determinations made from different cell preparations. See Fig. 2 legend and Materials and Methods for further details.

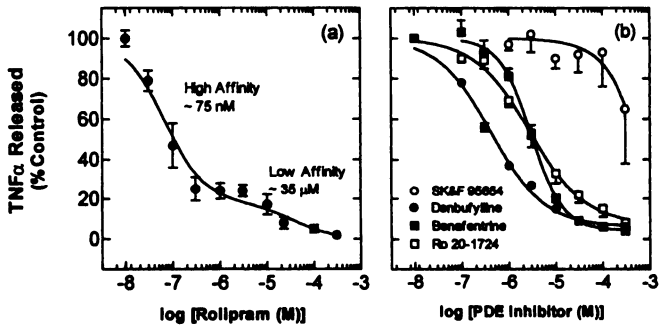

Fig. 5. Effect of cAMP PDE inhibitors on LPS-induced TNF-α biosynthesis from human peripheral blood monocytes. Adherent monocytes were pretreated for 20 min with varying concentrations of either (a) rolipram or (b) denbufylline, benafentrine, Ro 20–1724, or SK&F 95654 before being exposed to LPS (3 ng/ml; EC_{84}). Values represent the mean ± standard error of three determinations made from different cell preparations. See Fig. 2 legend and Materials and Methods for further details.

TABLE 1

Effect of cyclic nucleotide PDE inhibitors on LPS-induced TNF-α generation from human peripheral blood monocytes

Adherent monocytes were pretreated for 20 min with the PDE inhibitors indicated before being exposed to LPS (3 ng/ml). Cells were maintained at 37° in a thermostatically controlled incubator under a 5% CO_2 atmosphere, and the amount of TNF-α released into the culture supernatant was measured at 18 hr with a sandwich ELISA. Values represent the mean ± standard error of *n* determinations taken from different cell preparations. Values in parentheses represent the percentage inhibition achieved at the highest concentration of PDE inhibitor examined. See Materials and Methods for further details.

PDE inhibitor	*n*	Isoenzyme selectivity	IC_{50}	Slope
			μM	
Rolipram	3	PDE4	0.077 ± 0.015 (high) 33.60 ± 0.016 (low)	—
Denbufylline	3	PDE4/5	0.34 ± 0.07	0.749 ± 0.046*
Ro 20–1724	3	PDE4	3.18 ± 0.98	0.743 ± 0.035*
Benafentrine	3	PDE3/4	5.51 ± 2.37	1.258 ± 0.186
SK&F 95654	3	PDE3	>300 (35%)	—
Zaprinast	3	PDE1/5/6	>100 (19%)	—
MY 5445	3	PDE1/5/6	>30 (38.7%)	—
SK&F 96231	3	PDE1/5/6	>30 (10.2%)	—

* $p < 0.05$, slope significantly less than unity.
Values in parentheses indicate percentage inhibition at the highest concentration of PDE inhibitor examined.

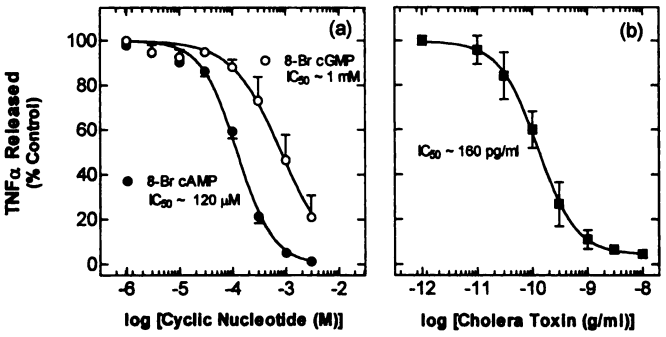

Fig. 6. Inhibitory effect of cell-permeant cyclic nucleotide analogues and of cholera toxin on LPS-induced TNF-α biosynthesis from human peripheral blood monocytes. Adherent monocytes were pretreated (a) for 20 min with varying concentrations of either 8-bromo-cAMP or 8-bromo-cGMP or (b) for 2 hr with cholera toxin before being exposed to LPS (3 ng/ml; EC_{84}). Values represent the mean ± standard error of three determinations made from different cell preparations. See Fig. 2 legend and Materials and Methods for further details.

pressive effect on cytokine production. Fig. 7 shows the inhibitory action of two β_2-adrenoceptor agonists, used clinically, on the generation of TNF-α. Exposure of human monocytes to albuterol or procaterol for 5 min before the addition of LPS resulted in a partial inhibition (~40%) of

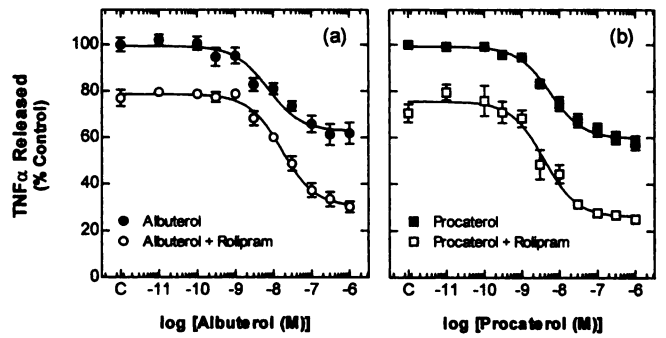

Fig. 7. Interaction of rolipram with the β_2-adrenoceptor agonists albuterol and procaterol on LPS-induced TNF-α biosynthesis from human peripheral blood monocytes. Adherent monocytes were pretreated for 15 min with 100 nM rolipram (●, ■) or its ethanol vehicle (○, □) and then for an additional 5 min with varying concentrations of (a) albuterol or (b) procaterol before being exposed to LPS (3 ng/ml; EC_{84}). Values represent the mean ± standard error of five determinations made from different cell preparations. See Fig. 2 legend and Materials and Methods for further details.

TNF-α production measured at 18 hr (Table 2). Procaterol was approximately equipotent with albuterol (EC_{50}: 7.7 versus 10.4 nM, respectively) and exhibited the same relative intrinsic activity (Fig. 7; Table 2). The inhibition of TNF-α generation evoked by albuterol and procaterol was prevented in monocytes that were pretreated with propranolol (1 μM), indicating that this effect was β-adrenoceptor mediated (Table 3).

A curious observation that emerged from these experiments was that neither of the β_2-adrenoceptor agonists studied interacted synergistically with rolipram at inhibiting TNF-α production (Fig. 7). Only an additive effect was observed when these drugs were used in combination. Thus, in the presence of 100 nM rolipram, which suppressed the elaboration of TNF-α by ~20%, the EC_{50} values for albuterol and procaterol were not significantly reduced with respect to control values (Table 2). Furthermore, although the maximum inhibition of TNF-α generation elicited by rolipram was

TABLE 2

Interaction between rolipram and charybdotoxin and the cAMP-elevating drugs albuterol, procaterol, PGE_2, and forskolin on LPS-induced TNF-α generation from human peripheral blood monocytes

Adherent monocytes were pretreated for 15 min with rolipram (100 nM), charybdotoxin (30 nM), or vehicle (0.0001% v/v ethanol) and then for an additional 5 min with albuterol, procaterol, or PGE_2, as indicated. Similarly, rolipram was added to monocytes for 10 min before the addition of forskolin (10 μM for 10 min). Monocytes were then exposed to LPS (3 ng/ml). Cells were maintained at 37° in a thermostatically controlled incubator under a 5% CO_2 atmosphere, and the amount of TNF-α released into the culture supernatant was measured at 18 hr with a sandwich ELISA. Values represent the mean ± standard error of *n* determinations taken from different cell preparations. See Materials and Methods for further details.

Treatment	*n*	EC_{50}	Maximum inhibition
		nM	%
Albuterol	3	15.2 ± 2.3	39.2 ± 4.9
Albuterol + rolipram	3	17.9 ± 2.4	76.9 ± 0.7
Albuterol + charybdotoxin	6	25.2 ± 4.1	25.3 ± 4.6
Procaterol	3	7.7 ± 2.9	37.0 ± 5.5
Procaterol + rolipram	3	4.4 ± 1.1	75.0 ± 1.2
PGE_2	3	66.7 ± 13.5	88.5 ± 2.2
PGE_2 + rolipram	3	25.9 ± 4.2*	94.5 ± 1.1
PGE_2 + charybdotoxin	5	70.3 ± 6.9	88.6 ± 2.6
Forskolin	3	No effect	10.4 ± 7.2
Forskolin + rolipram	3	No effect	26.7 ± 6.2

* $p < 0.05$, potency of PGE_2 + rolipram significantly higher than PGE_2 alone.

TABLE 3

Effect of propranolol on the inhibition of LPS-induced TNF-α generation from human peripheral blood monocytes evoked by albuterol and procaterol

Adherent monocytes were pretreated for 15 min with propranolol (1 μM) or its vehicle and then for an additional 5 min with either albuterol (1 μM) or procaterol (1 μM) before being exposed to LPS (3 ng/ml). Cells were maintained at 37° in a thermostatically controlled incubator under a 5% CO_2 atmosphere, and the amount of TNF-α released into the culture supernatant was measured at 18 hr with a sandwich ELISA. Values represent the mean ± standard error of *n* determinations taken from different cell preparations. See Materials and Methods for further details.

Treatment	*n*	TNF-α	
		−Propanolol	+Propanolol
		ng/ml	
Basal	3	0.19 ± 0.17	0.17 ± 0.15
LPS	3	1.54 ± 0.25	1.70 ± 0.31
Albuterol	3	1.29 ± 0.21*	1.64 ± 0.28†
Procaterol	3	1.28 ± 0.24*	1.78 ± 0.21†

* $p < 0.05$, significant inhibition of LPS-induced TNF-α generation.
† $p < 0.05$, significant antagonism of the inhibitory effect evoked by albuterol and procaterol on LPS-induced TNF-α generation.

increased by albuterol and procaterol, this was attributable solely to the inhibitory effect of rolipram (Fig. 7).

In view of the apparent lack of synergy between β_2-adrenoceptor agonists and rolipram and to assess in more detail the role that cAMP plays in the suppression of TNF-α production, similar studies were performed with a number of other agents that are reputed to enhance adenylyl cyclase activity. Fig. 8 shows the effect of PGE_2 and forskolin on LPS-induced TNF-α generation. Pretreatment of human monocytes for 5 min with PGE_2 potently inhibited cytokine generation in a concentration-dependent manner (EC_{50} = 67 nM). Compared with the results obtained with the β_2-adrenoceptor agonists, however, two main differences were apparent (Fig. 8a; Table 3). First, PGE_2 was significantly more effective than either albuterol or procaterol at inhibiting TNF-α generation (~90% versus ~40%, respectively, at their maximal effective concentrations). Second, rolipram (100 nM) potentiated the inhibitory effect of PGE_2. There was a statistically significant 2.6-fold decrease in the EC_{50} of PGE_2 in

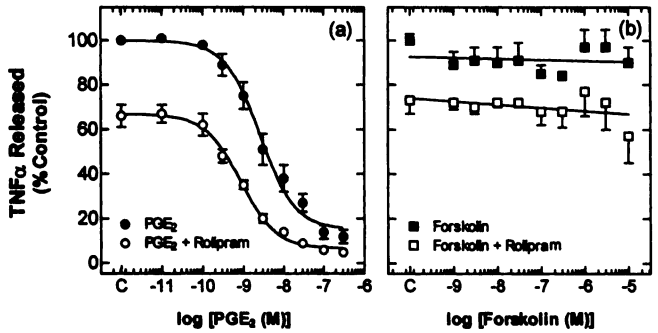

Fig. 8. Interaction of PGE_2 and forskolin with the PDE4 inhibitor rolipram on LPS-induced TNF-α biosynthesis from human peripheral blood monocytes. Adherent monocytes were exposed to varying concentrations of (a) PGE_2 or (b) forskolin in the absence (●, ■) and presence (○, □) of rolipram (100 nM) added 15 or 5 min before the addition of PGE_2 and forskolin, respectively. LPS (3 ng/ml; EC_{84}) was then added, and the concentration of TNF-α-like immunoreactivity released into the culture supernatant was measured at 18 hr with a sandwich ELISA. Values represent the mean ± standard error of three determinations made from different cell preparations. See Materials and Methods for further details.

rolipram-treated monocytes (from 67 to 26 nM; Table 2). In contrast, pretreatment of monocytes for 20 min with forskolin, a direct activator of adenylyl cyclase, failed to inhibit LPS-induced TNF-α generation at concentrations up to 10 μM regardless of whether the cells were preexposed to 100 nM rolipram (Fig. 8b; Table 2).

Effect of albuterol, PGE_2, and forskolin on cAMP levels: Interaction with rolipram. Fig. 9 show the effect of rolipram on the cAMP content of human monocytes and the interaction of this PDE4 inhibitor with albuterol, PGE_2, and forskolin. Exposure of monocytes to rolipram for 20 min did not increase the cAMP content at any concentration (100 nM to 100 μM) examined, suggesting that basal adenylyl cyclase activity was low in these cells (Fig. 9). Similarly, neither albuterol (1 μM for 5 min) nor PGE_2 (1 μM for 5 min) significantly increased the level of cAMP at concentrations that produced the maximal inhibition of LPS-induced TNF-α generation (Fig. 9, a and b). However, when albuterol and PGE_2 were added to monocytes that had been exposed to rolipram, a highly significant synergistic increase in the cAMP content was observed (Fig. 9,a and b). Furthermore, rolipram synergized more effectively with PGE_2 than with albuterol. In cells exposed to the highest concentration of rolipram studied (100 μM), PGE_2 increased the cAMP content approximately 8-fold (from 0.75 ± 0.22 to 6.31 ± 0.95 pmol/10^6 cells), whereas only a 3-fold increment in cAMP mass (from 1.21 ± 0.35 to 3.54 ± 0.81 pmol/10^6 cells) was effected by albuterol (compare Fig. 9a with Fig. 9b).

Exposure of human monocytes to forskolin (10 μM for 20 min) similarly failed to elevate the cAMP content but, in contrast to PGE_2 and albuterol, did not interact in a synergistic manner with rolipram at any concentration studied (Fig. 9c).

Effect of albuterol and PGE_2 on the PKA activity ratio: Interaction with rolipram. Fig. 10 illustrates the effect of rolipram on the activation state of PKA and the interaction of this PDE4 inhibitor with albuterol and PGE_2. In contrast to the cAMP data described, exposure of monocytes to rolipram for 20 min increased the PKA activity ratio in a concentration-dependent manner (from 0.11 ± 0.016 to 0.183 ± 0.036 at 100 μM rolipram, $p < 0.05$), with a mean EC_{50} of 1.1 μM. Similarly, albuterol (1 μM for 5 min) and PGE_2 (1 μM for 5 min) evoked a modest, but nevertheless statistically significant, increase in the PKA activity ratio to 0.153 ± 0.031 and 0.218 ± 0.036, respectively, under conditions where the total intracellular cAMP level was apparently unchanged (Fig. 10, a and b). When albuterol and PGE_2 were added to monocytes that had been preexposed to rolipram (10 nM to 100 μM), a highly significant synergistic increase in the PKA activity ratio was observed at all concentrations of rolipram examined (Fig. 10, a and b). Furthermore, in agreement with the cAMP data shown in Fig. 9, rolipram synergized more effectively with PGE_2 than with albuterol. In cells exposed to the highest concentration of rolipram studied (100 μM), PGE_2 increased the PKA activity ratio to 0.72 ± 0.029, whereas only a relatively small activation of PKA (to 0.33 ± 0.034) was noted with albuterol (compare Fig. 10a with Fig. 10b).

Effect of CbTX on the inhibitory action of albuterol and PGE_2 on LPS-induced TNF-α generation. Pretreatment of monocytes with charybdotoxin (30 nM for 20 min), an inhibitor of certain K^+ channels, including the high conduc-

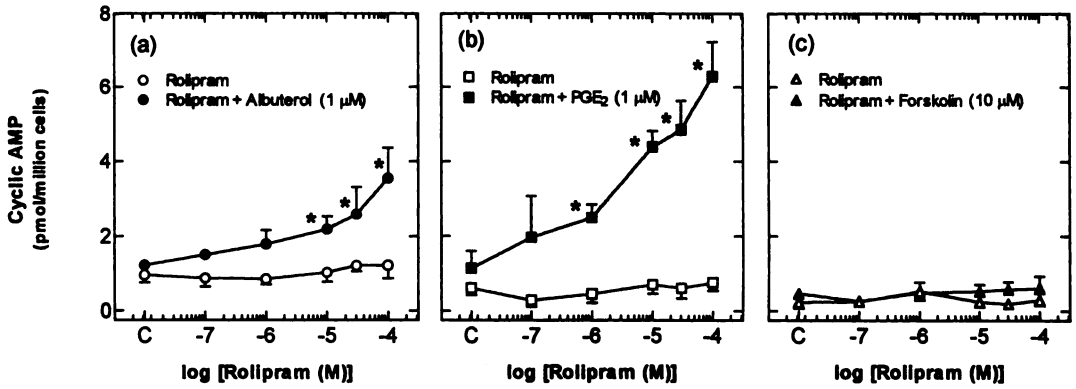

Fig. 9. Interactions of albuterol, PGE_2, and forskolin with the PDE4 inhibitor rolipram on the cAMP content of human peripheral blood monocytes. Adherent monocytes were exposed to (a) albuterol (1 μM for 5 min), (b) PGE_2 (1 μM for 5 min), or (c) forskolin (10 μM for 10 min) in the absence (●, ■) and presence (○, □) of varying concentrations of rolipram added 10 or 15 min before the addition of forskolin or albuterol and PGE_2, respectively. The reaction was stopped by the addition of boiling trichloroacetic acid (1 M), and cAMP was subsequently extracted, neutralized, and measured with radioimmunoassay as detailed in Materials and Methods. Values represent the mean ± standard error of three determinations made from different cell preparations. *, $p < 0.05$ for synergistic increase in cAMP mass between rolipram and adenylyl cyclase stimulant, one-way ANOVA/Newman-Keuls test.

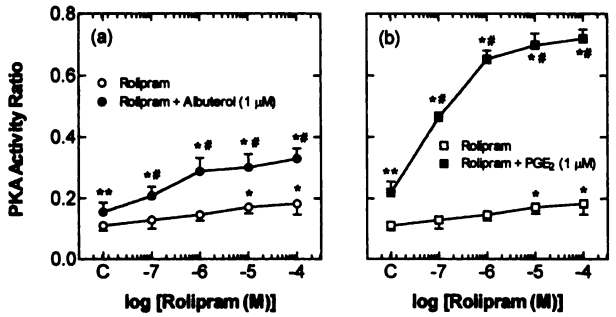

Fig. 10. Interaction of albuterol and PGE_2 with the PDE4 inhibitor rolipram on the PKA activity ratio of human peripheral blood monocytes. Adherent monocytes were exposed to (a) albuterol (1 μM for 5 min) or (b) PGE_2 (1 μM for 5 min) in the absence (●, ■) and presence (○, □) of varying concentrations of rolipram added 15 min before the addition of albuterol and PGE_2. The reaction was stopped by the addition of ice-cold buffer E, and the PKA activity ratio was subsequently estimated as described in Materials and Methods. Values represent the mean ± standard error of three determinations made from different cell preparations. *, $p < 0.05$ for synergistic increase in PKA activity ratio between rolipram and adenylyl cyclase stimulant, one-way ANOVA/Newman-Keuls test.

tance calcium-activated potassium channel, failed to antagonize the ability of albuterol to suppress LPS-induced TNF-α generation measured at 18 hr (Table 2). PGE_2-induced responses were similarly unaffected by charybdotoxin (Table 2).

Effects of SNP and ANP on LPS-induced TNF-α generation. Pretreatment of human monocytes for 5 min with SNP (1 nM to 1 mM) or ANP (0.1 nM to 10 μM) failed to affect the basal elaboration of TNF-α or that evoked by the subsequent addition of LPS at any concentration examined (data not shown).

Discussion

The results of the present study demonstrate that human peripheral blood monocytes in culture generate and release the proinflammatory cytokine TNF-α in response to bacterial LPS. This effect was dependent on new protein synthesis and was inhibited by a number of agents reputed to increase the cAMP content in intact cells. Intriguingly, evidence was obtained that β_2-adrenoceptor agonists suppressed LPS-induced TNF-α production by a mechanism that may be unrelated, at least in part, to the activation of the cAMP/PKA cascade.

The ability of LPS to increase the amount of immunoreactive TNF-α released into the culture medium was abolished in monocytes pretreated with actinomycin D or cycloheximide, indicating that LPS promotes transcription and translation of the TNF-α gene. There is compelling evidence that LPS, alone or combined with a specific serum-derived binding protein, interacts with CD14 on phagocytes to activate and/or induce a number of transcription factors, in particular, nuclear factor-κB, for which consensus sequences have been identified on the promoter region of the TNF-α gene (25). This interpretation is supported further by the findings that there was a lag period of at least 1 hr after the addition of LPS before TNF-α was detected in the culture supernatant and that cytokine production was also suppressed by dexamethasone, with an IC_{50} value in close agreement with its dissociation constant for the glucocorticoid receptor (26). Curiously, dexamethasone did not abolish LPS-induced TNF-α production, and ~20% of the cycloheximide/actinomycin D-sensitive response remained in steroid-treated monocytes. These results suggest that induction of TNF-α gene expression by LPS may involve several mechanisms, not all of which are sensitive to glucocorticosteroids.

With the use of classic biochemical and pharmacological techniques, the predominant PDE in human cultured monocytes was ascribed to the PDE4 isoenzyme family. Thus, cAMP hydrolysis was potently inhibited by Ro 20–1724 and other rolipram-like drugs but was unaffected by SK&F 95654 and by cGMP, suggesting that these cells do not express members of the PDE2 or PDE3 isoenzyme families. Close inspection of the data, however, revealed that cAMP hydrolysis was suppressed by only 80–95%, depending on the PDE4 inhibitor used, indicating that monocytes contain a residual activity that may represent the recently discovered PDE7. cAMP hydrolysis was stimulated to a small extent by the Ca^{2+}/calmodulin complex, demonstrating that these cells also contain a PDE1 isoform; PDE5 and PDE6, however,

were apparently absent as demonstrated from their inability to hydrolyze cGMP.

The identification of a PDE4 in human monocytes is consistent with recent findings that essentially all proinflammatory and immunocompetent cells express at least one representative of this isoenzyme family (16–19). Furthermore, the observation that rolipram potentiated albuterol- and PGE_2-induced cAMP accumulation and activation of PKA indicates an important functional role for PDE4 in intact cells (see below). It is noteworthy that human macrophages express a representative(s) of both the PDE4 and PDE3 isoenzyme families (27). This indicates that the progressive differentiation and maturation of monocytes are accompanied by the induction of a PDE3 gene and implies that the control of cyclic nucleotide homeostasis is more complex in macrophages.

A major finding of the present study was that inhibitors of PDE4 suppressed LPS-induced TNF-α generation from human monocytes with a rank order of potency in good agreement with their ability to inhibit PDE4-catalyzed cAMP hydrolysis. These results are entirely consistent with PDE4 being the major cAMP hydrolyzing activity in these cells and confirm and extend previous communications that document the ability of nonselective and PDE4-selective inhibitors to modulate TNF-α generation from murine (28) and human monocytes (15,29–32). The observation that cholera toxin and 8-bromo-cAMP also suppressed the production of TNF-α suggests that cAMP-dependent mechanisms have a central role in the expression of this cytokine. In keeping with this, it is established that a principal mode of action of cAMP in LPS-stimulated cells is to repress transcription of the TNF-α gene rather than to reduce the stability of TNF-α mRNA (33,34). Exactly how this process is controlled is unknown, but Spriggs *et al.* (35) speculated that cAMP may activate an inhibitory factor that recognizes a consensus sequence(s) for a cAMP-responsive element (associated normally with the activation of gene transcription) on the TNF-α gene promoter. Evidence is also available that cAMP can regulate TNF-α expression at a post-transcriptional level (36,37).

If PDE4 inhibitors do repress LPS-stimulated TNF-α gene expression via a cAMP-dependent process, then why did rolipram fail to increase the cAMP level in monocytes, even at high concentrations where cytokine generation was essentially abolished? At least two explanations for this anomaly are worthy of consideration that are not mutually exclusive. First, PDE4 may be compartmentalized in the cell such that rolipram increases cAMP in discrete intracellular loci that cannot be detected when total monocyte cAMP mass is measured. Second, it is conceivable that basal adenylyl cyclase activity is low in monocytes and that only a small, undetectable, but nevertheless functionally important, increment in cAMP is necessary to suppress TNF-α generation. Evidence to support both possibilities was provided by the finding that rolipram evoked a significant, concentration-dependent increase in the PKA activity ratio, implying that rolipram does increase cAMP in monocytes. Furthermore, rolipram markedly synergized with albuterol and PGE_2 in the generation of cAMP and in the activation of PKA.

Despite the suppressive effect of PDE inhibitors on TNF-α generation, a number of differences between the drugs examined in the present study were apparent. The concentration-response curves for denbufylline and Ro 20–1724 were shallow (or apparently biphasic in the case of rolipram), with pseudo-Hill coefficients significantly less than unity, whereas the inhibition curve for benafentrine was steep (slope ~1). Intriguingly, the functional findings with rolipram mirrored the shape of the concentration-response curves that described the inhibition of cAMP hydrolysis and, to some extent, the potentiation of albuterol- and PGE_2-induced cAMP accumulation. Similar effects have been described in a number of other cells and tissues, in particular, eosinophils (21,38), and may represent a drug-specific rather than a tissue-specific phenomenon. The reasons underlying this difference remain elusive but may relate to the fact that rolipram, denbufylline, and Ro 20–1724 preferentially interact with a conformation of PDE4 that is apparently different from that recognized by benafentrine-like compounds[1].

A number of investigators have reported that LPS stimulates PGE_2 production in macrophages and monocytes that can act in an autocrine manner to counter the associated biosynthesis of cytokine gene products, including TNF-α (24). In human cultured peripheral blood monocytes, however, no evidence was found to support this contention. Pretreatment of these cells with flurbiprofen at a concentration that inhibits the activity of both constitutive and inducible cyclo-oxygenases did not augment LPS-induced TNF-α generation, indicating that the release of endogenously synthesized cyclo-oxygenase products does not affect this response.

A number of reports have documented that exogenous PGE_2 effectively inhibits TNF-α generation and mRNA expression in macrophages and monocytes (e.g., Ref. 49) by a mechanism that involves activation of the cAMP/PKA cascade. Although the data presented in the present report confirm these observations, PGE_2 did not measurably increase the cAMP content in human monocytes at a concentration that produced the maximum inhibition of cytokine generation. Similar results were obtained with a high concentration of albuterol where the release of TNF-α was suppressed by ~40% in the apparent absence of a detectable increase in cAMP mass. One possible, albeit heretical, interpretation of these data is that cAMP does not mediate the inhibition of TNF-α generation by PGE_2 and albuterol. However, further experiments revealed that both agents significantly increased the PKA activity ratio, which provides unequivocal evidence that adenylyl cyclase was activated by both stimuli. These findings support the general consensus that measurement of the PKA activity ratio is a sensitive and superior index of changes in intracellular cAMP.

PGE_2 essentially abolished LPS-induced TNF-α generation from human monocytes under conditions where albuterol was only a relatively weak stimulus. This difference in effectiveness may relate, at least in part, to PGE_2 being a more robust activator of adenylyl cyclase in these cells than albuterol, which is supported by the finding that PGE_2 activated PKA to a greater extent than albuterol. Similarly, PGE_2-induced cAMP accumulation and PKA activation were markedly potentiated by rolipram relative to the same responses evoked by albuterol. Collectively, these data imply that the extent to which cAMP is elevated and PKA is activated is a primary determinant for the inhibition of LPS-induced TNF-α generation in human monocytes. This does not preclude, however, the participation of other cAMP-inde-

[1] J. J. Kelly, P. J. Barnes, and M. A. Giembycz, unpublished observations.

pendent pathways in the regulation of this response (see below).

A particularly perplexing observation was the inability of rolipram to potentiate the inhibitory influence of albuterol and procaterol on LPS-induced TNF-α generation. Classically, these data are contrary to the fundamental pharmacological tenet that states that inhibitors of cyclic nucleotide PDEs should act synergistically with activators of adenylyl and guanylyl cyclase. These findings, however, are not peculiar to the human monocyte. Torphy *et al.*, for example, reported that rolipram failed to potentiate isoproterenol-induced relaxation of human bronchial smooth muscle (40). Furthermore, rolipram interacts in a purely additive manner with albuterol in the inhibition of hydrogen peroxide generation from human eosinophils but, nevertheless, potentiates albuterol-induced cAMP accumulation (21). One explanation for this paradox is that β_2-adrenoceptor agonists increase the cAMP content in a subcellular compartment that is inaccessible to the rolipram-sensitive PDE. In this scenario, inhibition of PDE4 would have no effect on albuterol-induced cAMP accumulation or on the PKA activity ratio. However, rolipram and albuterol interacted synergistically in the activation of the cAMP/PKA cascade, indicating that PDE4 does have access to the cAMP generated by β_2-adrenoceptor agonists. Alternatively, PDE4 may not represent the major regulator of cAMP in monocytes. Again, this seems unlikely given that PDE4 was the predominant cAMP hydrolyzing activity expressed in cultured cells and that the cAMP level and PKA activity ratio were increased in a more-than-additive manner when rolipram and albuterol were used in combination. Another possibility is that albuterol-induced cAMP accumulation is also regulated by PDE1 and that this isoenzyme regulates TNF-α generation. Finally, it is also conceivable that β_2-adrenoceptor agonists elicit functional effects in cells by a mechanism(s) separate from, or in addition to, the generation of cAMP.

Perhaps the best characterized cAMP-independent action of β-adrenoceptor agonists is their ability to increase the open state probability of the high conductance calcium-activated potassium channel (41). This is believed to be mediated via a stimulatory G protein and results in membrane hyperpolarization and a consequent reduction in cell excitability. In human monocytes, however, albuterol did not apparently use this biochemical pathway because charybdotoxin, a potent and well-characterized inhibitor of these channels (41), failed to antagonize the inhibition of LPS-induced TNF-α generation.

Another curious result was the failure of forskolin to inhibit LPS-induced TNF-α generation. This was particularly unexpected given that PDE4 inhibitors, 8-bromo-cAMP and cholera toxin effectively suppressed cytokine production by a mechanism that probably relies on the activation of the cAMP/PKA cascade. The reason for this anomalous result presumably relates to the inability of forskolin to increase the cAMP content in monocytes even at concentrations (e.g., 10 μM) that produce a marked activation of adenylyl cyclase in other cells and tissues. Although beyond the scope of the present study, at least two explanations could account for this result. The first and most simplistic is that forskolin is unable to cross the plasmalemma and gain access to the interior of the cell. Clearly, the direct measurement of adenylyl cyclase activity in monocyte plasma membranes will resolve this issue. The second possibility is that monocytes express an abundance of an adenylyl cyclase variant(s) that is insensitive to forskolin. This is an attractive postulate because evidence is available for at least eight adenylyl cyclase variants that have been categorized into five isoenzyme families (42). These cyclases differ not only structurally but also in tissue distribution, subcellular location, and, importantly, mode of regulation (42). It is entirely plausible, therefore, that adenylyl cyclase isoenzymes exist that are insensitive to forskolin.

A final point for discussion concerns the effect of cGMP on TNF-α biosynthesis. Neither ANP nor hybrid inhibitors of PDE1, PDE5, or PDE6 affected basal or LPS-induced cytokine generation. These data are thus entirely consistent with the lack of PDE5 and PDE6 in, and the absence of receptors of ANF on, human monocytes (43). Similarly, SNP, which stimulates soluble guanylyl cyclase in human monocytes (43), had no effect on basal and LPS-elicited TNF-α production, and 8-bromo-cGMP was only a weak inhibitor, which may be due to cross-activation of PKA (44). Collectively, these data suggest that cGMP does not play a major role in regulating TNF-α biosynthesis from freshly prepared human monocytes. Inexplicably, however, these observations are at variance with those obtained from a number of other studies. Molnar-Kimber *et al.* (29) reported that zaprinast (PDE1/PDE5/PDE6 inhibitor) significantly augmented the production of TNF-α from LPS-stimulated human monocytes, implying that cGMP-dependent mechanisms can positively regulate TNF-α gene expression. A similar conclusion was reached by Gong *et al.* (45) based on the finding that SNP was more effective than LPS at evoking TNF-α generation from murine peritoneal macrophages. Further experiments are clearly required to reconcile these inconsistent findings.

In conclusion, the primary observation of the present study was that drugs that inhibit PDE4 activity effectively prevented the production of TNF-α from human peripheral blood monocytes, probably by repressing TNF-α gene expression (34). This is an important observation because (a) it extends the pharmacological actions of PDE4 inhibitors to include suppressing the deleterious actions of a chronic proinflammatory mediator that is implicated in the pathogenesis of a multitude of disorders, including arthritis, asthma, and, possibly, multiple sclerosis; and (b) it strengthens the current postulate that PDE4 inhibitors may have therapeutic potential as novel anti-inflammatory agents (16–19). Finally, the results challenge the well-established doctrine that β_2-adrenoceptor agonists exert their functional effects solely by elevating intracellular cAMP.

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